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Analysis of immunoglobulin and T cell receptor gene expression in ballan wrasse (*Labrus bergylta*) revealed an extraordinarily high IgM expression in the gut

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ABSTRACT

The serum IgM concentration of ballan wrasse is relatively high, estimated to approximately 13 mg/ml in adult wild fish of 800 g. The present study revealed an unusual high abundance of IgM mRNA in the gut of ballan wrasse. Initially, transcripts encoding IgM, IgT, IgD, TCRα, TCRδ and CD3ε were quantified by RT-qPCR in several tissues of wild caught fish (approx. 800 g), indicating an elevated immune activity in hindgut and an extraordinarily high expression of IgM. Subsequently, a new RT-qPCR analysis was performed on the entire intestine, cut into four different segments, of reared fish (32–100 g). The analysis indicated immune activity along the entire intestine, but not as strong as in the hindgut. Furthermore, similar to the larger fish, the relative abundance of IgM transcripts was higher in the hindgut than in kidney and spleen, although the absolute level of IgM was in general higher in the larger fish. The secreted form of IgM was completely dominant in comparison to the membrane bound form of IgM and the other analysed genes. IgM was purified from gut mucus and external mucosal surfaces by magnetic beads coated with protein A. Mucus IgM reacted with rabbit antisera raised against serum IgM and contained subunits of the same size. Regarding the elevated immune activity in the intestine it is tempting to speculate on a possible compensatory strategy in this lineage of stomach-less fish, and that natural antibodies have an important role in the first line defence.

1. Introduction

Most teleosts have three immunoglobulin (Ig) classes, IgM, IgD and IgT/IgZ, defined by the heavy chains of the molecules μ, δ and τ, respectively. IgM is the main systemic antibody, usually present as tetramers. IgT appears to have a role in systemic immune responses as well as in mucosa [1,2]. The IgT molecules were found as monomers in serum of rainbow trout whereas in gut mucus they were tetramers [2]. The constant region of IgD shows a remarkable structural diversity in teleosts. The functional role of IgD is still somewhat enigmatic. In catfish (*Ictalurus punctatus*), IgD was proposed to act as a pattern recognition molecule [3] and in the gills of rainbow trout (*Oncorhynchus mykiss*) it functions in maintaining microbiotal homeostasis [4]. In another study of rainbow trout, the IgD response against viral infection was found to be weak [5]. Studies of Nile tilapia (*Oreochromis niloticus*), rohu (*Labeo rohita*) and freshwater carp (*Catla catla*) showed upregulation of IgD transcripts in immune organs after viral, bacterial and parasitic infections [6–8].

The concentration of total IgM in serum vary to a large extent among teleost species, and also according to several other parameters, like age/size, gender, season, environment and vaccination/infection status (Hordvik, 2015). The serum IgM concentration was found to be 13 mg/ml in adult wild ballan wrasse (approx. 800 g) and showed high binding affinity to protein A (Bilal et al., 2016). Protein A is commonly used for affinity purification of some immunoglobulin isotypes in mammals. Protein A affinity to IgM varies among teleosts, from almost no binding in for example salmonids to high binding in other species [9–12].

As in other jawed vertebrates, the T cells in teleost fish are of either α/β or γ/δ type. The T cell receptors (TCR) are arranged in a complex with CD3 molecules on the cell surface [13,14]. Typically, teleosts possess CD8 and CD4 co-receptors which interact with MHC class I and class II antigens, respectively [25,46]. As in higher vertebrates, the TCRα and TCRδ genes are linked in the genome of teleost fish,

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including ballan wrasse (*Labrus bergylta*) (Bilal et al., 2018).

A characteristic feature of ballan wrasse is their lack of stomach and their small intestine, which is only about two third of the body length [15]. The stomach is characterized by the secretion of gastric juice (HCl and pepsinogen) and evolved early in gnathostome ancestors about 450 million years ago. The acidic environment of the stomach allowed jawed vertebrates to use a broader range of dietary protein sources over the course of evolution. The low pH of the stomach facilitates the denaturation of large proteins and subsequent digestions by endopeptidases, uptake of calcium and phosphate, and provides a first line defence by acting as a barrier against pathogen entry [16]. The secondary loss of stomach has occurred independently in many jawed vertebrates, including lamprey, hagfish, chimaeras, some teleosts, lungfish and monotremes. Among teleosts, the examples of agastric fish include fugu (*Takifugu rubripes*), green spotted pufferfish (*Tetraodon nigroviridis*), zebrafish (*Danio rerio*), medaka (*Orzias latipes*), common carp (*Cyprinus carpio*), garfish (*Belone belone*) and hairychin goby (*Sagamia geneionema*). The analysis of genomic data from different gnathostome lineages suggests a correlation between loss of stomach and absence of pepsinogens and gastric proton pump genes (ATP4A and ATP4B) [17–19]. Accordingly, whole genome sequencing of ballan wrasse revealed loss of genes associated with gastric function [47].

Ballan wrasse is an important cleaner fish in salmon aquaculture, but very few studies have been published on the immune system of this species [12,20,21]. In the present work, RT-qPCR assays were developed for key markers of the adaptive immune system (IgM, IgT, IgD, TCRα, TCRδ and CD3ε) and transcription was measured in different organs. The RT-qPCR showed extraordinarily high IgM transcript levels in hindgut. The abundance of mRNAs encoding secreted and membrane bound Ig was studied in more detail and IgM was purified from gut and external mucosal surfaces using magnetic beads coated with protein A.

2. Methods

2.1. Fish samples

Adult, wild ballan wrasses (600–930 g) were caught in May and June from fjords near Bergen, Norway. Fish were killed with a sharp blow to the head and tissue samples (head kidney, spleen, gut, gill, liver, muscle) were collected in the field and stored in RNA-later solution (Ambion). From the adult, wild ballan wrasses, additional samples from the four different gut segments (see Fig. 1) and gills were transferred to 4% buffered formalin and stored at 4 °C until further processing for immunohistochemical investigation. In a second sampling, five ballan wrasse (32–100 g) reared in tanks and fed commercial feed were obtained from the Institute of Marine Research (IMR), Austevoll Research Station, Norway. Mucus samples were collected from wild catch fish transported in water tanks to the dissection laboratory.

2.2. RNA isolation and cDNA synthesis

RNA was isolated from tissues of ballan wrasse using TRIzol® reagent (Invitrogen). The quantity of total RNA was measured using a Nanodrop spectrophotometer. For cDNA synthesis 400 ng of RNA was used in a total reaction volume of 20 µl. First strand cDNA was synthesized using SuperScript™ II reverse transcriptase (Invitrogen) and an oligo dT_{16} primer.

2.3. PCR-amplification of cDNA, molecular cloning and DNA sequencing

Primers for IgM, TCRα and TCRδ were designed from sequences described previously (Bilal et al., 2016; Bilal et al., 2018). IgT, IgD, CD3ε and EF1α primers were based on intestinal transcriptome data and ballan wrasse genome sequences (European Nucleotide Archive accession number: PRJEB13687). Primer sequences are given in Table 1. Amplification with Accuprime™ High Fidelity Taq DNA polymerase (Invitrogen) was performed as follows: denaturation at 94 °C for 2 min, 30 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (30 s), and extension at 68 °C (1 min/1000 bps). The cDNA fragments were excised from gel, and purified DNA was cloned into pCR™ 4-TOPO® vector (Invitrogen). Sequencing was performed at an in-house sequencing facility using Big Dye termination chemistry (Applied Biosystems).

2.4. Sequence analysis and phylogeny

DNA/protein sequences were compared to the GenBank/EMBL databases using BLAST. DNA was translated into amino acid sequence using the translate tool at ExPasy https://web.expasy.org/translate/. Multiple sequence alignments were performed using MUSCLE https://www.ebi.ac.uk/Tools/msa/muscle/. The phylogenetic trees were constructed using MEGA6 software and neighbour joining (NJ) with 1000 boot-strap replicates [22]. The putative N-glycosylation and O-glycosylation sites were predicted using online servers: NetNGlyc 1.0 http://www.cbs.dtu.dk/services/NetNGlyc/ and NetOGlyc 4.0 respectively http://www.cbs.dtu.dk/services/NetOGlyc/.

2.5. Reverse transcription quantitative PCR (RT-qPCR)

Initially, the abundance of IgM, IgT, IgD, TCRα, TCRδ, CD3ε and

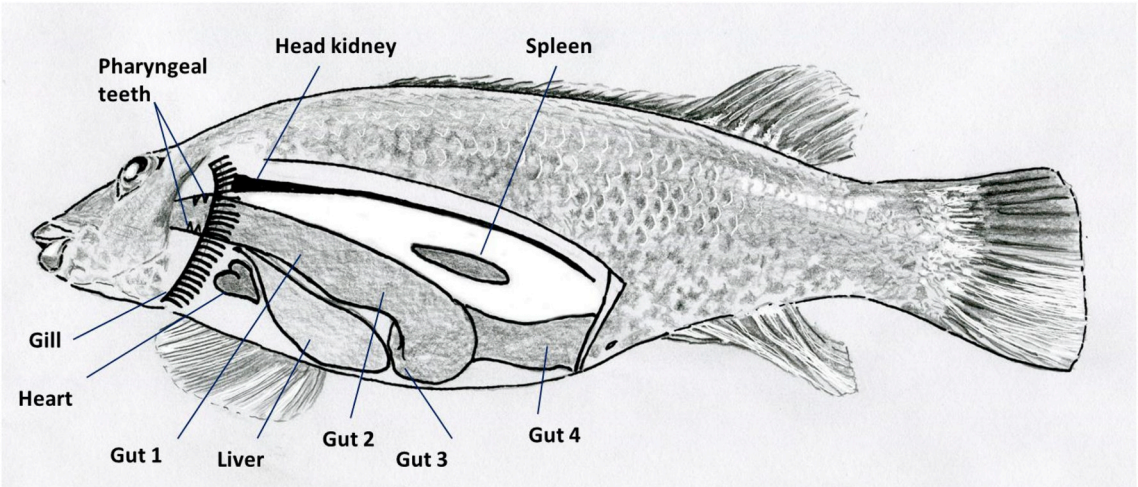

Fig. 1. Ballan wrasse, a stomach-less fish with a short intestine. The tissues which were collected for RT-qPCR analysis and immunohistochemistry are indicated.

Table 1
Primers, probes and percentage efficiencies for TaqMan and SYBR Green assays.

Assay	Primers and probes	Sequence 5′→ 3′	Position	% Efficiency
IgM	Forward	GAGCAACAATGGGCTCAACA	μ2	101
TaqMan	Probe	TGCGAGGAAATTCCAACAAGTCCCGT	μ2-μ3	
	Reverse	CGTGACCTCACACACCATTG	μ3	
IgD	Forward	TGACATGGAGGCTGGATGAG	δ2	97
TaqMan	Probe	TCCTTCAGTCTTTGCCAGCTGCTTG	δ2	
	Reverse	ACTGCAGGTCCTGGAACAAA	δ2-δ3	
IgT	Forward	CTCAGAGTGGCAGACAGTCA	τ3	98
TaqMan	Probe	ACCGTCCACGTCCTCCCAGAGG	τ4	
	Reverse	TGACCTCGTTTCCTTTGCTG	τ4	
TCRα	Forward	GATATCCGACGGGTCTGTGT	Cα	99.5
TaqMan	Probe	AGCTGGCCGCCAATTCATCACA	Cα-CP	
	Reverse	AGGATCTCCACAGTCACCAG	CP	
TCRδ	Forward	GAACCTACGCCGTTGAAACC	Cδ	100
TaqMan	Probe	AGCTGAACGACGCCTCCACCA	Cδ	
	Reverse	GTCACAGGTGTCGACTTTGG	CP	
CD3ε	Forward	CTCAACCCTCTGGTGTTTGC	TM	102
TaqMan	Probe	ACGCCGTCCCGATCACGTCCA	TM	
	Reverse	CAGGTGCTGTGGAAGCATTG	TM-CYT1	
EF1α	Forward	ATTGATGCCCCTGGACAC	Ex3	98.4
TaqMan	Probe	CTGCGCTGTGCTGATCGTTGC	Ex 4	
	Reverse	CCTCAAACTCACCGACACC	Ex4	
sIgM	Forward	AATATTGGAGGACTGACTCAGCG	μ3- μ4	93
SYBRGreen	Reverse	CGTCATCAACAAGCCAAGACACA	μ4	
mIgM	Forward	GACCCCATCAAGAAACAGCTTG	μ3	104.4
SYBRGreen	Reverse	GGCAGGTTCGGCCATGTTATC	TM	
sIgD	Forward	ACCGGGAGCAGCACTTATTC	δ6	92.3
SYBRGreen	Reverse	ACAGCTCACCAAACACATCCTT	δ6-Sec	
mIgD	Forward	ACCGGGAGCAGCACTTATTC	δ6	94.7
SYBRGreen	Reverse	GCTCTTCAATACGTCAAACACA	δ6-TM	
sIgT	Forward	ACCTCAGAAGACCCAACAAGG	τ4	98.4
SYBRGreen	Reverse	GTTACCCAACTCACTTTTGACTCC	τ4-Sec	
mIgT	Forward	GCCCAGGAGTCAAAAGTGAGT	τ4	98.4
SYBRGreen	Reverse	GGAATATGAAAGAGGCGGCTG	TM	
EF1α	Forward	ATTGATGCCCCTGGACAC	Ex-3	96.8
SYBRGreen	Reverse	CCTCAAACTCACCGACACC	Ex-4	

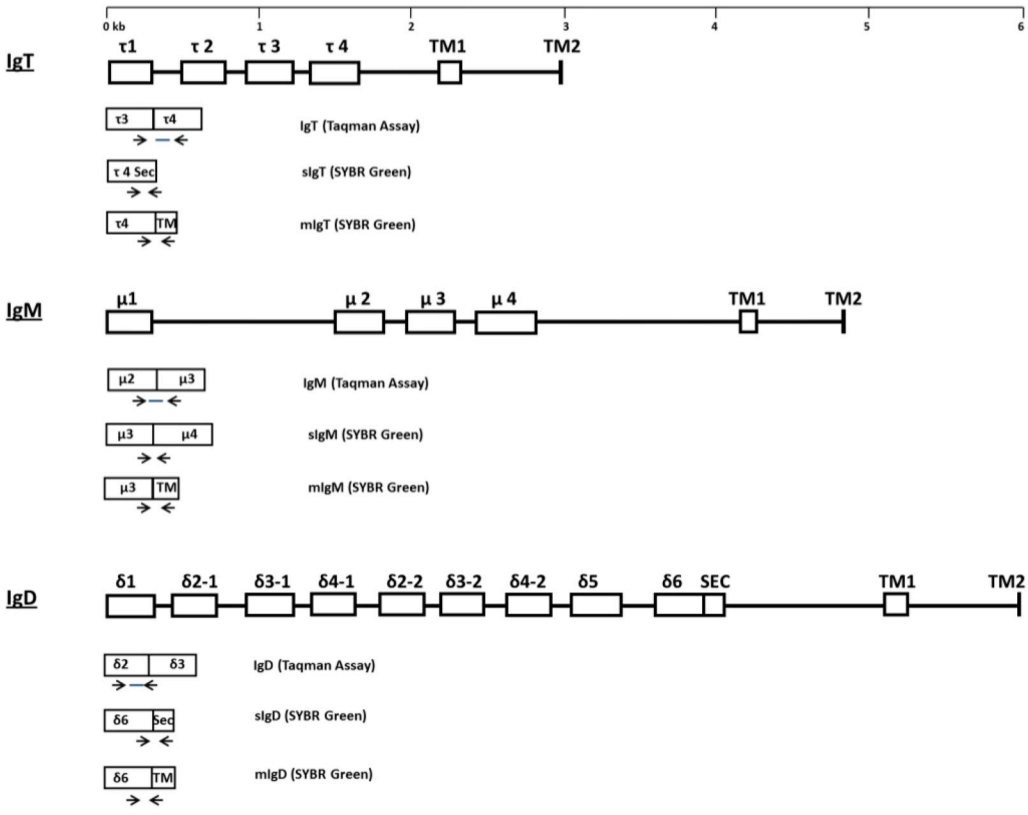

Fig. 2. Gene structures of IgT, IgM and IgD in ballan wrasse. Gene structures are based on scaffold LaB_20160104_scaffold_1388. The positions of primers and probes for RT-qPCR assays are indicated with arrows. Boxes in the figure show exons while introns are represented by straight lines. Sizes are drawn to scale as indicated by the scale bar.

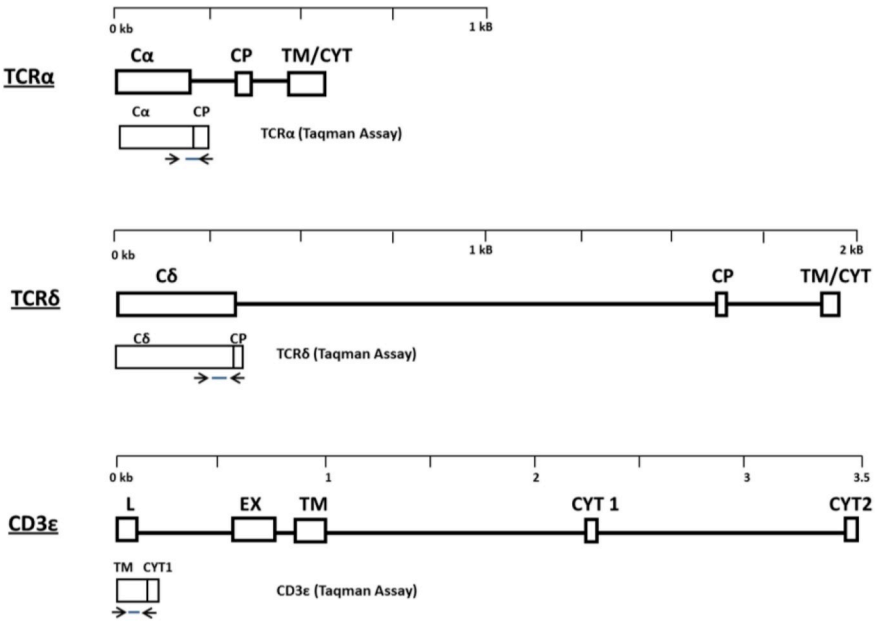

Fig. 3. Gene structures of CD3ε and the constant regions of TCRα and TCRδ in ballan wrasse. Gene structures for TCRα and TCRδ are based on LaB_20160104_scaffold_928 from the ballan wrasse genome database. The CD3ε gene structure is based on LaB_20160104_scaffold_217. The position of primers and probes for RT-qPCR assays are indicated with arrows. Boxes in the figure show exons while introns are represented by straight lines. Sizes are drawn to scale as indicated by the scale bar. L: signal peptide, EX: extracellular domain, CP: connecting peptide, TM: transmembrane region, CYT: cytoplasmic region.

EF1α mRNAs was analysed by TaqMan® MGB probe-based qPCR assays using the QuantStudio™ 3 Real-Time PCR System (Applied Biosystems™). The assays were designed in such way that primers or probe should be in two different exons (Fig. 2 and Fig. 3). The efficiencies of all TaqMan® assays were evaluated by making series dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) from a pool of cDNA from different tissues, and ranged from 97 to 102% (Table 1). The qPCR reaction mixture contained: TaqMan™ Universal PCR Master Mix (2×), 900 nM of each primer, 250 nM of TaqMan probe and 4 µl of 1:10 diluted cDNA (cDNA from each tissue was synthesized using 400 ng of RNA) in a final volume of 10 µl. The amplification reaction conditions were 95 °C for 10 min and 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing/extension). All samples were run in triplicates with no-template controls (NTC). The EF1α gene was used as reference gene and IgT from muscle was used as calibrator.

SYBR Green assays were used for quantification of splice variants of IgM, IgD and IgT. The primer pairs and efficiencies of the assays are given in Table 1. The qPCR reactions were run in a total volume of 10 µl, containing SYBR® Select Master Mix (2×), forward and reverse primers (400 nM each) and 2 µl of 1/10 diluted cDNA. The amplification conditions were as follows: UDG (Uracil-DNA glycosylase) activation at 50 °C for 2 min, AmpliTaq ® Fast DNA Polymerase UP activation at 50 °C for 2 min followed by 40 cycles of 95 °C for 15 s (denaturation) and 60 °C for 1 min (annealing/extension). Melting curves from 65 °C to 95 °C were added at the end. Samples were run in triplicates with no-template controls (NTC). IgT from gill was used as calibrator. The relative expression of different transcripts was calculated by using the double delta Ct method. EF1α was used as endogenous control to normalize the expression.

2.6. Statistical analysis

The transcript abundance of Ig and T cell marker genes among different tissues was compared by one-way ANOVA followed by Tukey's test, with values < 0.05 as significantly different. The difference between mean values of secreted and membrane form of Ig genes was compared by using student's T-test (2-tailed).

2.7. Purification of IgM from gut and external mucosal surfaces

Mucus from external surfaces (skin, gills, and nasopharynx) was collected by keeping the fish in a plastic bag for approximately 5 min with 6 ml of PBS pH 7.4 mixed with protease inhibitor mini tablets (Pierce™). Mucus from gut was collected by opening the intestine longitudinally and incubating it with PBS, pH 7.4, mixed with protease inhibitor. Mucus samples were centrifuged at room temperature for 15 min at 2000 × *g* to get rid of tissue and scales. The supernatant (400 µl) from gut and skin mucus samples were incubated with 50 µl of protein A coated Dynabeads (30 mg/ml) for 10 min at room temperature with rotation. After binding, the supernatant was removed and beads were washed three times with PBS (pH 7.4) 0.02% Tween®-20. Beads were eluted with 1 × SDS sample buffer and boiled for 5 min before the supernatant was applied on gel.

2.8. SDS-PAGE and western blot analysis

The protein samples were run on reducing, denaturing, 4–15% gradient gels. Western blotting was performed at 60 V for 60 min at 4 °C. The PVDF membrane was blocked in 5% dry milk in PBS for 1 h and incubated overnight at 4 °C with rabbit serum against ballan wrasse IgM (1:1000 dilution in PBS). Rabbit immune sera against ballan wrasse IgM was produced by a 63 day protocol with five immunizations (Davids Biotechnologie GmbH). The membrane was washed 3 × in PBS-Tween (0.02%) at room temperature for 5 min each and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:5000 dilution in PBS). The PVDF membrane was developed using ECL reagents (Pierce™ ECL Western Blotting Substrate).

2.9. Immunohistochemistry

Formalin-fixed tissues of different gut segments (Fig. 1) and gills (second gill arch) were processed, paraffin-embedded and sectioned at 4 µm thickness followed by mounting on poly-L-lysine-coated slides (KF FROST, VWR International BV, Amsterdam, The Netherlands) using standard procedures [23]. The slides were incubated at 37 °C for 24 h, followed by 58 °C for another 24 h, before deparaffinization in xylene and hydration in graded ethanol dilutions to distilled water. Heat-induced epitope retrieval was performed at 121 °C for 10 min in 0.01 M citrate buffer, pH 6. The slides were subsequently washed in 0.01 M phosphate-buffered saline (PBS, pH 7.3). Inhibition was obtained by incubation for 40 min at 37 °C in preheated PBS with 0.05% phenylhydrazine (Merck KGaA, Darmstadt, Germany), followed by washing with PBS and blocking for 20 min in 0.05 M tris-buffered saline (TBS, pH 7.6) with 0.2% lyophilized normal goat serum (ImmunO MP

Biomedicals, Solon, OH, USA) and 5% bovine serum albumin (BSA) (Biotium, Fremont, CA, USA). Polyclonal ballan wrasse IgM primary antibody [12] was diluted 1:10 000 in TBS with 1% BSA before application, and the slides were incubated overnight at 4 °C. After rinsing with PBS, the slide were incubated with polymer-HRP anti-rabbit (Dako EnVison + System-HRP, Dako, Glostrup, Denmark) and developed with 3-amino-9-ethyl carbazol (AEC) as substrate, producing a red reaction product. Between each step, the slides were rinsed in TBS. Counterstaining was performed with Mayer's haemalum solution (Merck KGaA, Darmstadt, Germany) followed by washing in distilled water and mounted with FluorSave Reagent (Merck KGaA, Darmstadt, Germany). As negative control, primary antibody was omitted from the procedure.

3. Results

3.1. IgM, IgT and IgD gene structures in ballan wrasse

IgT and IgD sequences from Atlantic salmon were used as queries to search for counterparts in an intestinal transcriptome database of ballan wrasse. IgM (as previously described), IgT and IgD cDNA sequences were further used to identify the genes in the recently assembled genome of ballan wrasse in GenBank. The BLAST searches revealed many scaffolds comprising Ig sequences.

The scaffold LaB_20160104_scaffold_1388 (43413 bps) contained typical IgT, IgM and IgD genes, and an organization similar to other teleosts. The IgT gene consisted of four Cτ exons and two transmembrane exons (TM1 and TM2). The IgM gene comprised four Cμ exons and two TM exons (TM1 and TM2). The IgD gene, located immediately downstream of IgM, consisted of six unique Ig domain exons of which δ2, δ3 and δ4 were duplicated, i.e. with an exon organization like δ1-δ2.1-δ3.1-δ4.1-δ2.2-δ3.2-δ4.2-δ5-δ6-TM1-TM2. The percentage nucleotide identities between different duplicated domains varied; i.e. δ2.1 and δ2.2 had only 60% identity, δ3.1 and δ3.2 had 99.3%, while δ4.1 and δ4.2 shared 94% sequence identity. The structures of the genes and position of primers and probes for real time PCR is shown in Fig. 2.

3.2. TCRα, TCRδ and CD3ε gene structures in ballan wrasse

The genes encoding TCRα and TCRδ were identified in LaB_20160104_scaffold_928 [21]. Salmon CD3ε was used as query to find the ballan wrasse counterpart in the transcriptome database, and subsequently the gene was identified in LaB_20160104_scaffold_217. The structures of the genes and position of primers and probes for real time PCR are shown in Fig. 3.

Ballan wrasse TCRδ cDNAs were cloned and sequenced (GenBank accession number: MH802588). An amino acid alignment with other teleost species showed that the constant Ig domain (Cδ) and connecting peptide of ballan wrasse TCRδ is highly diverged whereas the transmembrane and cytoplasmic regions are more conserved. The second cysteine residue in Cδ, which is usually involved in intra-chain disulphide bonding, is replaced by a tyrosine (Y) residue. Many putative O-glycosylation sites in Cδ showed high predictability indices. The O-glycosylation stabilizes the structure of proteins by increasing the rigidity [24] and is assumed to be important for an extended stalk structure in several co-receptors, like CD8 [25]. The cysteine residue in the connecting peptide region is suggested to form inter-chain disulphide bond in the heterodimer formation. The TCRδ alignment of ballan wrasse and phylogenetic relationship to other species is shown in supplementary Fig. 1.

3.3. Immunoglobulin and T cell marker gene expression in tissues of ballan wrasse

The abundance of Ig and T cell marker mRNAs was measured by RT-qPCR in different tissues of adult wild caught ballan wrasse (Fig. 4). Initially, the assays were designed to detect all isoforms of each gene, respectively. IgM gene expression was generally very high compared to the other studied genes. Among the different tissues analysed, IgM showed an unusually high expression in hindgut (for example 2.9 times higher than in head kidney). There was a significant difference between IgM transcript levels in different tissues (supplementary document 1).

For IgT, the highest expression was found in spleen. The levels were similar in kidney and hindgut, followed by gill, liver and muscle. IgD transcripts were most abundant in spleen followed by kidney, hindgut and gill. A statistically significant difference was found in the IgT and IgD expression among different tissues as shown in supplementary document 1. The abundance of immunoglobulin transcripts were in the order: IgM ≫ IgT > IgD. The ratio of IgM/IgT and IgM/IgD in different tissues is given in supplementary Table 1.

TCRα transcript levels were highest in spleen and hindgut, followed by gill and kidney. For TCRδ highest levels were found in hindgut followed by gill, spleen and kidney. For CD3ε the highest expression was also found in hindgut, followed by spleen, gill and head kidney. TCRα, TCRδ and CD3ε showed significant difference in their expression among different tissues (supplementary document 1).

3.4. Transcript levels of immunoglobulin and T cell markers in different parts of the gut

After observing high levels of transcripts in hindgut of wild fish (approximately 800 g), the abundance of transcripts was analysed in different parts of the gut in individuals kept in tanks and fed commercial feed (fish with average weight of 62 g). The gut was numbered in ascending order, foregut (part after pharynx) as gut 1 and hindgut as gut 4 (Fig. 1). For IgM, IgT and TCRα highest levels were found in the hindgut, while for TCRδ and CD3ε there was not much difference in expression in different parts of the gut. The abundance of IgD transcripts was lower in the first segment compared to the last three segments of the gut (Fig. 5). However, no statistically significant difference was found between the transcript levels of immunoglobulin and T cell marker genes among different parts of the gut (supplementary document 2).

3.5. Transcript levels of different splice variants of immunoglobulin molecules

The transcript levels of secreted and membrane bound forms of IgM, IgD and IgT were analysed in head kidney, spleen, hindgut and gill. Transcription of the membrane bound form of IgM is minor compared to the secreted form. For IgT the levels of secreted and membrane forms are similar in each tissue. The membrane bound form of IgD is more abundant than the putative secreted form in the tissues studied (Fig. 6). Statistically significant differences between mean values of secreted and membrane forms of immunoglobulin genes is shown in supplementary document 3 and by asterisks on Fig. 6.

3.6. Affinity purification of IgM from gut and external mucosal surfaces

IgM from gut and external mucosal surfaces was purified utilizing magnetic beads coated with Protein A. SDS-PAGE analysis showed heavy and light chain bands at 75 kDa and 25 kDa, and Western blot analysis showed reactivity with rabbit sera against ballan wrasse serum IgM (Fig. 7). Serum IgM was purified as a positive control.

3.7. Distribution of IgM-positive cells in the gut and gills

From immunohistochemical staining, high numbers of IgM-positive cells were revealed within the epithelial lining of the gut (Fig. 8A–D). While the content of blood vessels in the underlaying connective tissue (lamina propria) stained intensively with the IgM-antibody (probably targeting secretory IgM), very few IgM-positive cells were seen within this compartment of the gut mucosal lining (Fig. 8D). Furthermore,

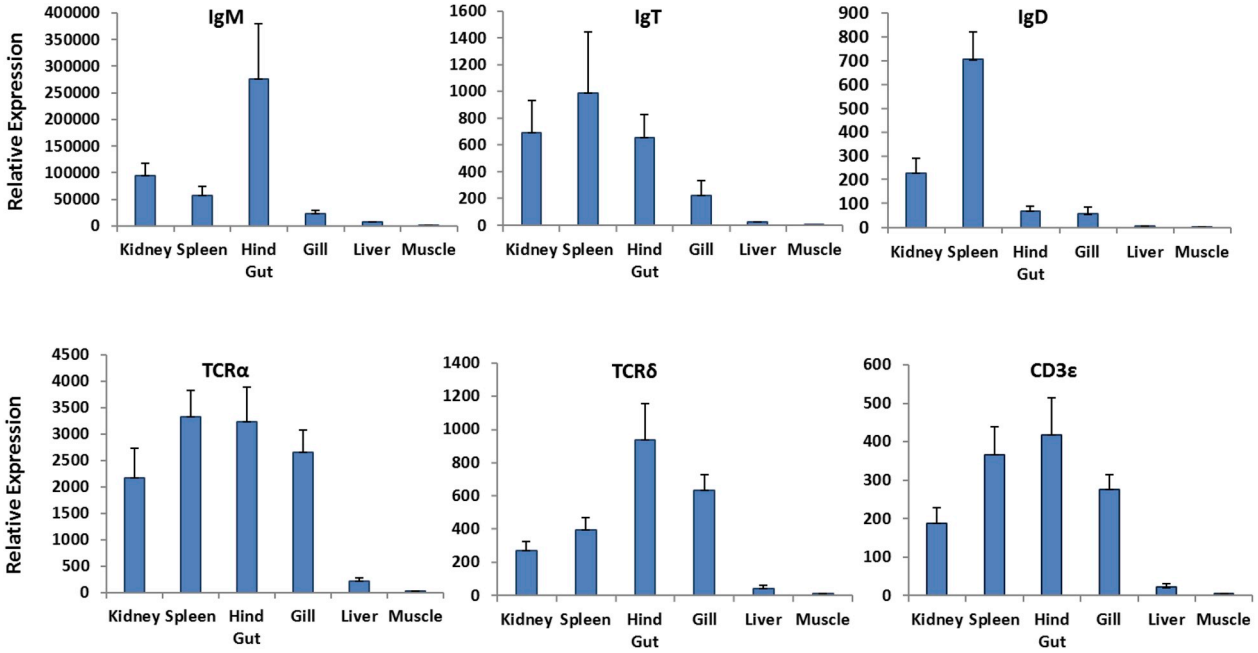

Fig. 4. The relative abundance of IgM, IgT, IgD, TCRα, TCRδ and CD3ε in different tissues, as measured by RT-qPCR. The TaqMan assays measured the overall expression of the genes. EF1α was used as reference gene for normalization of data, and IgT from muscle was used as calibrator. Data represent mean values of n = 8 for head kidney, gill, liver and muscle and n = 7 for spleen and hindgut of wild-caught ballan wrasse. Bar indicates the mean ± SEM.

from a qualitative perspective, higher numbers of IgM-positive cells were seen in the epithelial lining in the posterior segment (Gut 4 in Fig. 1) compared with the anterior segment (Gut 1 in Fig. 1) (Fig. 8B versus Fig. 8A, respectively). Compared with the gills, another important immunological mucosal tissue, only few IgM-positive cells were seen in the epithelial lining while the content of blood vessels stained intensively similar to what was seen for the blood vessels in lamina propria of the gut (Fig. 8E).

4. Discussion

The present work revealed a remarkably high IgM gene expression and presence of IgM-positive cells in the intestine of ballan wrasse. Typically for teleosts, IgM expression is highest in kidney which is the main haematopoietic organ, and spleen which is major secondary lymphoid [26–30]. In ballan wrasse, IgM mRNAs were more abundant in hindgut than in head kidney and spleen. The expression in gut was

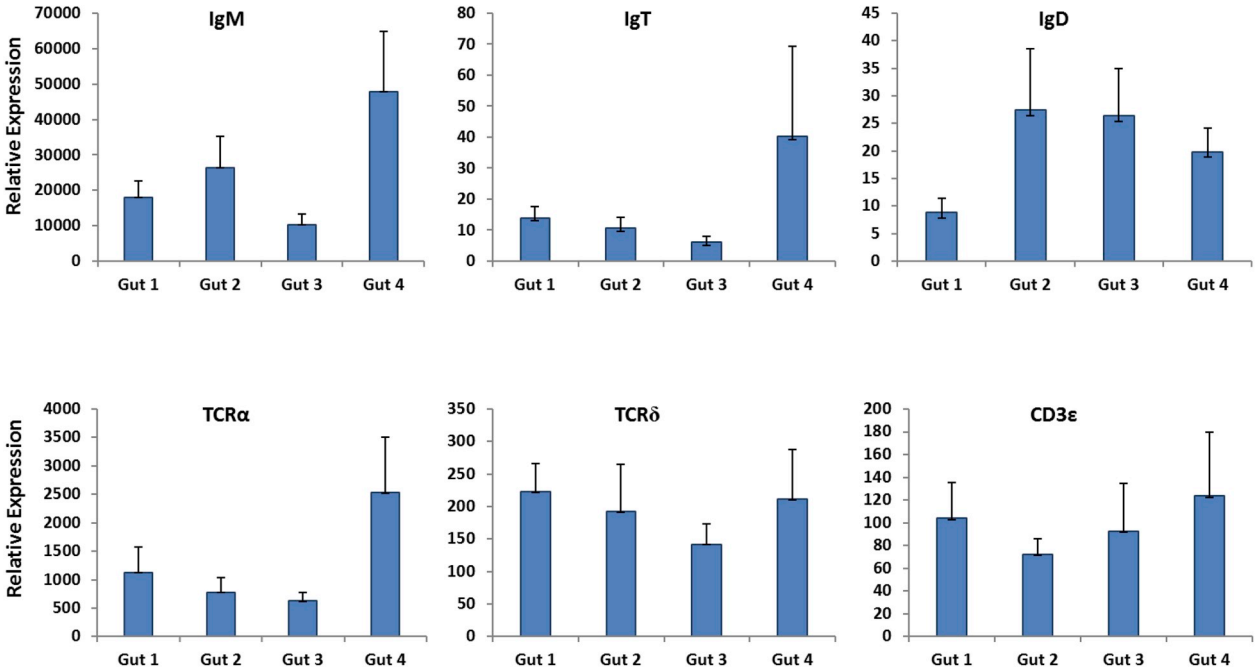

Fig. 5. Abundance of IgM, IgT, IgD, TCRα, TCRδ and CD3ε mRNA in different parts of the gut, measured by RT-qPCR. The gut number starts from foregut (1) and proceed to hindgut (4). The TaqMan assays measured the overall expression of the genes (both secreted and membrane bound forms). EF1α was used as reference gene for normalization of the data and IgT from muscle was used as calibrator. Data represent mean values (± SEM) of n = 5 for IgM, IgT, TCRδ, CD3ε and n = 4 for IgD and TCRα. The individuals were ballan wrasse kept in tanks and fed commercial feed.

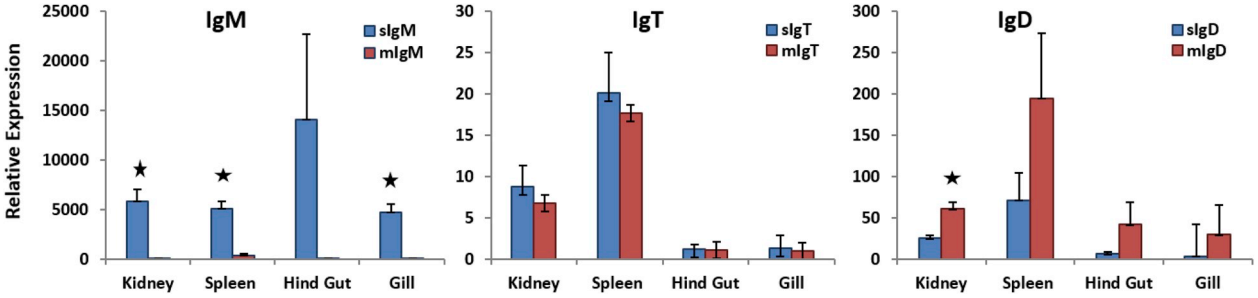

Fig. 6. Relative expression of secreted and membrane bound forms of IgM, IgT and IgD in different tissues, as measured with SYBR Green assays. EF1α was used as reference gene for normalization of data and IgT from gill was used as calibrator. Data represent mean values (± SEM) of n = 4 individuals of farmed ballan wrasse. sIgM, sIgT, sIgD: secreted forms; mIgM, mIgT, mIgD: membrane bound form. Asterisks represent statistically significant differences between the means of secreted and membrane forms. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

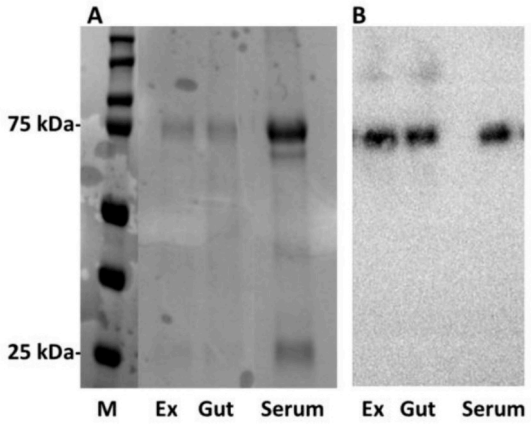

Fig. 7. Purification of IgM from external mucosal surfaces (Ex) and gut mucus of ballan wrasse. Serum was used as positive control. A) Protein A purified samples separated by SDS-PAGE and stained with Coomassie blue. B) Rabbit sera against ballan wrasse IgM showed reactivity with mucus IgM on corresponding Western blot (approximately equal amounts of mucus and serum IgM were applied on the gel in Fig. 7B).

highest in wild caught ballan wrasse (approx. 800 g), but the same relative trend was observed between tissues in smaller fish given commercial feed in tanks (average weight 62 g). As expected, transcripts encoding the secreted form of IgM were entirely dominant in comparison to the other studied genes. The relative levels of TCRδ, TCRα and CD3ε in head kidney, spleen and hindgut indicated an elevated immune activity in the gut of adult wild fish.

Incubation of mucus from gut and external mucosal surfaces with protein A coated magnetic beads purified molecules with sub-units of same size as serum IgM. The mucosal IgM reacted with rabbit antisera raised against ballan wrasse serum IgM (Fig. 7). In a previous report, the serum concentration of IgM was estimated to 13 mg/ml in adult wild caught ballan wrasse [12]. This is for example ten times higher than a typical IgM concentration in wild Atlantic salmon [31]. In cod, which have high IgM concentrations and show weak specific antibody responses, it has been proposed to be related to the lack of MHC class II genes [32,33]. This is not an explanation in ballan wrasse which possess both MHC class II and CD4 genes (results not shown). The finding of an extraordinarily high expression in the gut of ballan wrasse reflects a generally high level of secreted IgM in the body and an elevated immune activity in the gut compared to other teleosts. In hindgut, transcripts encoding secreted IgM were 258 times more abundant than the membrane bound form in ballan wrasse. In comparison, in rainbow trout the highest IgM expression was found in spleen where secreted IgM levels were approximately 5.4 times higher than membrane IgM [28]. Interestingly, a semi-quantitative analysis of common carp, which is also agastric, showed a relatively strong IgM expression in the intestine of unstimulated fish [34]. However, the typical pattern of higher IgM expression in spleen and kidney was found in other carp species [35].

The secreted and membrane forms of IgT were more equally expressed in the analysed tissues. Wild ballan wrasse of 800 g had relatively more IgT in gut than smaller fish kept in tanks. In 800 g fish the IgT mRNA level was highest in spleen and similar in kidney and gut. The basal IgT mRNA level in other species has shown diverse patterns. In Atlantic salmon the highest IgT expression was found in kidney followed by spleen while in rainbow trout the highest level was in spleen followed by blood, kidney, gut and gills [26,30]. In sea bass (*Dicentrarchus labrax*), gut and gills, important mucosal organs showed high IgT transcripts levels followed by kidney and spleen [36]. In rainbow trout, IgT protein levels were twice as high in gut mucus (7.1 μg/ml) as compared to serum, but still quite low as compared to IgM levels in the gut (74.9 μg/ml) and serum (2520 μg/ml). In response to parasitic infection, IgT gene expression was strongly upregulated in gut and there were more IgT^+ cells [1,2]. Although basal IgT mRNA levels in gut of ballan wrasse are relatively low, IgT might have a similar role in mucosal immune responses as in other teleosts.

Ballan wrasse IgD showed highest expression in spleen, followed by kidney, hindgut and gills. The membrane form of IgD was more abundant than the putative secreted form. However, only the difference between secreted and membrane form in kidney was statistically significant. The IgD gene in ballan wrasse consists of six unique Ig domain (Cδ) exons and two TM exons. The secreted form was predicted from transcriptome data, as a continuous read-through of δ6 until a stop-codon downstream of the splice donor site (adding a secretory tail consisting of 44 amino acids). It is common among teleosts that the membrane form of IgD is dominant, and secreted IgD, if present, is generated by different ways. In channel catfish and pufferfish (*Fugu rubripes*), membrane and secretory forms of IgD are encoded by two separate genes [37,38]. In rainbow trout the secreted form of IgD is generated by continuous read-through of the last Cδ exon [28].

In ballan wrasse, the highest TCRδ levels were found in hindgut and gills followed by spleen and kidney, suggesting a possible role in mucosal immunity. TCRδ is expressed as a heterodimer with TCRγ on the surface of γδ T cells. The γδ T cells are not MHC restricted and recognize unconventional antigens such as lipids and phosphorylated microbial metabolites [39]. In mammals, the γδ T cells play an important role in mucosal immunity [40]. In a study of zebrafish, the γδ T cells acted as antigen presenting cells and induced the production of IgM and IgT by T helper activation, primarily in the mucosal tissues intestine, gill and skin [41]. In Atlantic salmon, RT-PCR showed TCRα gene expression in head kidney, spleen and gills [42], whereas TCRδ expression was highest in gills as compared to other immune related tissues [43]. The transcripts of TCRα were found to be more abundant than TCRδ in all immune organs of ballan wrasse. The αβ T cells are called the conventional T cells and are generally more abundant than γδ T cells. CD3ε which is an important T cell marker was expressed in all immune

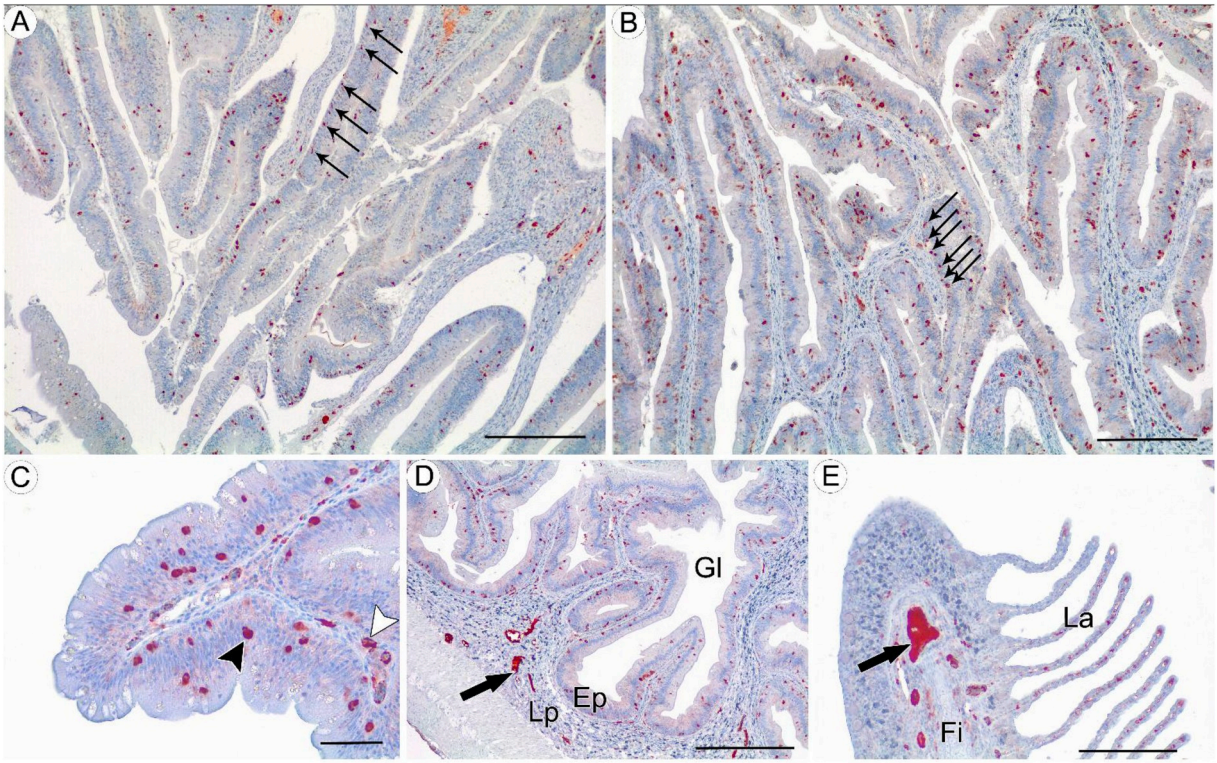

Fig. 8. Immunohistochemical staining against IgM in gut and gill of ballan wrasse. Binding of IgM is indicated by red reactions in the tissue sections. A) Epithelium of the anterior segment of the gut (Gut 1 in Fig. 1); arrows points to examples of IgM-positive cells. B) Epithelium of the posterior segment of the gut (Gut 4 in Fig. 1); arrows points to examples of IgM-positive cells. C) Higher magnification of immune-reaction in the intestinal folds of the posterior gut (Gut 4); black arrowhead points to an intraepithelial IgM-positive cell, while the white arrowhead points to an IgM-positive cell within the underlaying connective tissue (lamina propria). D) Section through the intestinal wall including surrounding muscular layers, lamina propria (Lp), epithelium (Ep) and gut lumen (Gl). Arrow points to IgM-staining of content within a blood vessel. E) Gill filament (Fi) and lamellae (La). Bold arrows point to IgM-staining of content within a blood vessel. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

related organs with the highest expression in hindgut of ballan wrasse.

The unusual high expression of IgM in hindgut, prompted us to investigate the expression of immune marker genes in all parts of the gut. The gut is not only involved in feeding, absorption, water and electrolyte balance, it is an important immunological site acting as a barrier against invading pathogens. TCRδ and CD3ε showed a uniform expression in all gut segments, while TCRα transcripts were more abundant in the hindgut of ballan wrasse. However, no statistically significant differences were found between different segments of gut for any immunoglobulin or T cell marker genes. In sea bass (*Dicentrarchus labrax*), microarray and qPCR profiling revealed a gradual increase in the immunological functions from anterior to posterior intestinal segments [44]. In Atlantic salmon, TCRα, TCRδ, IgM and IgT were most abundant in the second segment of the mid-intestine and the posterior segment [45].

The most surprising finding in the present study was the elevated immune activity in the intestine of ballan wrasse, and the extraordinarily high IgM expression and high number of IgM-positive intraepithelial cells. It is tempting to speculate whether this immune activity is related to the lack of stomach in ballan wrasse. Loss of stomach might have been compensated for in different ways during evolution, depending on the ecological niche each agastric teleost lineage has adapted to. In teleosts stomach has been lost in 20–27% of total species in more than 15 independent evolutionary events [16]. The immunoglobulins secreted in the mucosal tissues are important in maintaining the homeostasis and pathogen clearance at the boundary of the external environment. Thus, it is plausible to assume that a high concentration of natural antibodies could provide a stronger first line defence in mucosal barriers of ballan wrasse.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.02.007.

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